BISPECIFIC SINGLE-CHAIN FV MOLECULES THAT TARGET HER2/NEU AND HER3 MODULATE SIGNAL TRANSDUCTION

Eva M Horak, Tara Heitner, Deborah A Altomare, Jennifer L Garrison, Lillian S Shahied, Calvin C Shaller, Abohawariat Tesfaye, Heidi H Simmons, R Katherine Alpaugh, Nathaniel B Greer, Joseph R Testa, James D Marks, Louis M Weiner, and Gregory P Adams

Fox Chase Cancer Center, Philadelphia, PA;
University of California, San Francisco, San Francisco, CA

em_horak@fccc.edu

Signal transduction through members of the EGFR Family (EGFR, HER2/neu, HER3 and HER4) is dependent upon the formation of homodimers or heterodimers triggered by the binding of ligand. Overexpression of the members of this receptor family has been correlated with a poor prognosis in a number of types of cancer. Antibodies (such as Herceptin (anti-HER2), C225 and ABX-EGF (anti-EGFR) or small molecules like IRESSA, that perturb signaling through these receptors, have been associated with significant clinical responses. We hypothesize that bispecific single-chain Fv (bs-scFv) that bind to selected pairs of these receptors could prevent ligand induced signaling and trigger cytostatic or cytotoxic effects. We have previously described the production of the extracellular domains of EGF, HER2/neu, HER3 and HER4 receptors and their use as targets for selection of specific binders from a naive human scFv phage display library. We are now producing single gene bs-scFv molecules from these four sub-libraries with the goal of functionally selecting bispecific scFv molecules that target epitope pairs and mediate anti-tumor effects.

Our initial focus has been on HER2/neu and HER3. We have created a bs-scFv, ALM (comprised of the A5 and ML3.9 scFv molecules), that is capable of simultaneously binding to both the HER3 and HER2/neu receptor extracellular domains immobilized on a BIAcore chip. In biodistribution studies performed in an immunodeficient mouse model, radioiodinated ALM specifically targeted human BT-474 breast tumors that overexpress both targets. In in vitro assays, incubation with ALM led to a reduction in the level of HER3 and HER2/neu on the surface of BT-474 tumor cells and altered the phosphorylation of AKT-2 kinase, a major determinant in tumor cell survival.

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RECOMBINANT SCFV-ENZYME FUSION PROTEINS FOR ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

Deckert P. M., Petrausch U., Welt S., Bertino J., and Old L. J.

Hämatologie und Onkologie, Universitätsklinikum
Benjamin Franklin, Freie Universität,
12200 Berlin, Germany

deckert@medizin.fu-berlin.de

In ADEPT, tumor tissue is first targeted by an enzyme-linked antibody that recognizes a tumor antigen; in the second step, an inactive prodrug is administered and activated locally by the enzyme component. In theory, such systems should provide an ideal way of combining immunological specificity with the efficacy of chemotherapeutic agents, but poor diffusion of complete IgG-based antibody-enzyme-conjugates and their poor definition and stability have hitherto hindered the progress of this strategy. We have proposed an ADEPT system based on recombinant fusion proteins of a single chain fragments of tumor-binding antibodies such as F19, 3S193 or, used here as a model system, A33 with the enzyme cytosine deaminase (CD), using 5-fluorocytosine as a prodrug. Expression of such fusion proteins in inclusion bodies in E. coli requires extensive resolubilization and refolding procedures with low yield. To overcome these obstacles, we have established an expression system in methylotrophic Pichia pastoris yeast to produce soluble fusion proteins in culture supernatant.

DNA encoding the scFv, followed by a linker and the DNA encoding either CD or green fluorescent protein (GFP) were generated by RT-PCR and cloned in-frame into the pPIC-9k vector (Invitrogen). Cells from two different Pichia pastoris strains, GS115 and KM71, were then transformed with these vector constructs by electroporation. Based on the kanamycin resistance-gene of the host vector, we selected for genomic multicopy integrants. Resistant clones were then analysed by PCR for genomic integration of the target DNA. Positive clones were selected for protein expression in 50 ml pilot cultures by methanol induction under defined conditions for 96 hours.

Positive supernatants were dialysed against PBS and further purified by size exclusion filtration. Fluorescence microscopy showed binding of scFv-GFP to antigen-positive, but not to antigen-negative cell lines. These results were reproduced by flow cytometry. Here, pre-incubation with the A33-CD protein inhibited subsequent A33-GFP binding quantitatively. The function of the complete A33-CD-based ADEPT system was then demonstrated in vitro by cytotoxicity assays.

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ADOPTIVE THERAPY OF BREAST CANCER USING EFFECTOR LYMPHOCYTES TARGETED WITH HER/2-SPECIFIC CHIMERIC RECEPTORS

Zelig Eshhar, Dinorah Morvinski, Alain Bendavid, Tova Waks, and Daniel G. Schindler

Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel

zelig.eshhar@weizmann.ac.il

A major problem that impedes the application of active vaccination for cancer immunotherapy is that many tumor cells, including breast tumors, often escape the immune system. In order to overcome this difficulty, and to expand the recognition spectrum of effector lymphocytes and redirect them to breast cancer cells, we have endowed T and natural killer cells with antibody-type specificity, using chimeric receptor (CR) genes. Several configurations of CR have been designed to optimize the performance of the CR. To render such genetically programmed lymphocytes (we nick named "T-bodies") specific to breast cancer, we took advantage of the availability of HER/2-specific mAbs and used their scFv as recognition units of the CR. In order to achieve full T cell activity against adenocarcinoma that fail to express B7-type molecules, we designed a novel tripartite CR that combines both signaling domains of lymphocyte-triggering receptors and the CD28 co-stimulatory molecules. Primary T cells, derived from transgenic mice harboring such tripartite receptors indeed underwent full activation even by plastic immobilized antigen. Importantly, such cells did not undergo 'activation induced cell death', primarily because of the anti-apoptotic signal delivered by the CD28 moiety of the tripartite receptor. Using an efficient protocol for T cell transduction, employing the pBULLET retrovector pseudotyped with the GALV envelope, a high expression frequency of chimeric receptor genes has been obtained in human T cells (40-80%), starting from peripheral blood lymphocytes. Human lymphocytes expressing such tripartite CR appeared very efficient and specific effector cells in-vitro, undergoing cytokine release (IFN gamma, TNF alpha, IL-2) and mediating breast cancer cell lysis. To study the ability of such cells to eliminate existing human tumors, we made use of the SCID mouse system in which human breast cancer xenografts can readily grow. In such a system HER/2-specific T-bodies completely eliminated sub-cutaneously growing breast cancer xenografts. Systemic application of IL-2 is important factor in obtaining a long lasting effect. The models described serve now to study and establish the optimal conditions towards the application of the T-body approach to clinical trials.

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IMMUNOTHERAPEUTIC STRATEGIES IN A MOUSE MODEL OF SPONTANEOUS BREAST CANCER

Sandra J. Gendler, Pinku Mukherjee, Gargi D. Basu, Teresa L. Tinder, Cathy A. Madsen, and A. Renee Ginardi

Mayo Clinic Scottsdale, Departments of Tumor Biology and Biochemistry and Molecular Biology, 13400 East Shea Boulevard, Scottsdale, AZ 85259

gendler.sandra@mayo.edu

Immunotherapy for cancer is a promising non-toxic alternative to traditional therapies including chemotherapy and radiotherapy regimens that are often ineffective and harsh. Cancer vaccines are designed to target specific tumor antigens or unidentified tumor antigens, with the goal of stimulating a powerful immune response, leading to eradication of tumor cells by cytotoxic T cells (CTLs) and other effectors. One target tumor antigen is MUC1, which is over-expressed by more than ninety percent of tumors of the breast. Our objective is to develop an optimal breast cancer vaccine that will elicit anti-tumor immunity. Our specific aims are: 1) to assess the effectiveness of several MUC1 and/or tumor-specific vaccine formulations in the prevention and treatment of spontaneous breast carcinomas in mice and 2) to translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden.

To carry out these aims, we developed a mouse line that expresses human MUC1 as a self-molecule and spontaneously develops MUC1-expressing tumors of the mammary gland. Of key importance is that the tumors arise spontaneously and exhibit metastasis, similar to the human situation.

In these mice we tested a vaccine formulation comprised of liposomal-MUC1 lipopeptide and human rIL-2. Immunized mice developed T cells that a) express intracellular IFN-γ, b) are reactive with MHC class I H-2D^b/MUC1 tetramer, and c) are cytotoxic against MUC1-expressing tumor cells *in vitro*. However, the presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden or survival. Several tumor evasion mechanisms are utilized by these mice, including decreases in IFN-γ-expressing effector T cells, expression of immunosuppressive factors such as TGF-β2, and down-regulation of surface MHC class I molecules.

Although MUC1-specific CTLs are effective against tumor cell lines expressing MUC1, it is possible that spontaneous mammary tumor heterogeneity of expression may allow MUC1 non-expressing cells to grow out and form tumors. Thus, we are testing DC-based vaccines presenting a variety of tumor antigens by using 1) DCs fused with primary tumor cells and 2) DCs pulsed with tumor lysate. These immunization strategies were able to break tolerance and elicited a partial response including a significant increase in MUC1-specific CTLs. Using an injectable tumor model, we found that MUC1.Tg mice immunized with DC pulsed with tumor lysates were completely protected from subsequent challenge with the tumor cells. The challenge is to achieve these results in our spontaneous model. The vaccine most effective in the preclinical model will be translated into a Phase I clinical trial in breast cancer patients with low tumor burden in 2004.

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NEW STRATEGY FOR BREAST CANCER THERAPY: COMBINED SU6668 ANTIANGIOGENIC AND B7.2-IgG IMMUNE THERAPY OF LOCAL AND METASTATIC 4T1 BREAST TUMOR

Gorelik E.,[1] Wong M.,[1,2] Yi H.,[1] Watkins S.,[3] Laird A.D.,[4] Wolf S.F.,[5] and Huang X.[1]

Departments of [1]Pathology and [2]Medicine, [3]Center for Biological Imaging, University of Pittsburgh Cancer Institute, Pittsburgh, PA; [4]SUGEN, Inc., San Francisco, CA; and [5]Wyeth Research/Genetics Institute, Inc., Cambridge, MA

gorelik@pitt.edu

The therapeutic efficacy of combined antiangiogenic and immune therapy was tested against the highly invasive, weakly immunogenic and highly metastatic 4T1 breast tumor. Studies were performed using an angiogenesis inhibitor SU6668 and a murine recombinant B7.2-IgG fusion protein for immunostimulation. SU6668 is a novel inhibitor of the tyrosine kinase activity of three angiogenic receptors VEGFR2 (Flk-1/KDR), FGFR and PDGFR that play a crucial role in tumor-induced vascularization. B7.2-IgG is a homodimer of extracellular domain of recombinant murine B7.2 fused with the hinge and Fc portion of murine IgG2a. Immunomodulatory effect of B7.2-IgG could be a result of B7.2 interaction with CD28 and stimulation of T cells and/or with CTLA4 molecules and blocking suppression of T cell responses. Our studies showed that 3 weekly immunizations of BALB/c mice bearing 0.5 -0.8 cm 4T1 breast tumors with rmB7.2-IgG (100μg) and irradiated 4T1 tumor cells (B7.2-IgG/TC) resulted in a significant inhibition of tumor growth and formation of the pulmonary metastases. T cell depletion experiments revealed that stimulation of antitumor and antimetastatic immune response by B7.2-IgG/TC required both $CD4^+$ and $CD8^+$ T lymphocytes. Treatments with SU6668 (75 mg/kg, s.c. once a day) for 3 weeks did not inhibit the ability of T lymphocytes to respond to B7.2 stimulation. Although SU6668 inhibited vascular formation in 4T1 tumor, it did not prevent tumor infiltration by T cells. In fact, in tumors from mice that received combined SU6668 and B7.2-IgG therapy the numbers of CD4 and $CD8^+$ T lymphocytes were higher than in tumors treated separately with SU6668 and B7.2-IgG/TC. The therapeutic efficacy of combined SU6668 and B7.2-IgG/TC therapy has been tested against established (0.5-0.8 cm) 4T1 tumor using two different schedules. In first type of experiments daily treatments with SU6668 were started on day 3 and B7.2-IgG/TC immunizations were applied on days 12, 19 and 26. In another experiments immunizations were started on day 7 and repeated on days 14 and 21. SU6668 treatments were initiated on day 10 (3 days after first immunization). SU6668 treatments initiated either on day 3 or day 10 of tumor growth resulted in a significant and equal inhibition of tumor growth. Similarly, three weekly immunizations with B7.2-IgG/TC starting either on day 7 or day 12 inhibited growth of 4T1 tumors, although tumor inhibition was more profound when immunizations were initiated on day 7 than on day 12. However, the most potent antitumor effects were observed in mice treated with a combination of SU6668 and B7.2-IgG/TC. Analysis of pulmonary metastases revealed that combined therapy also had a more potent antimetastatic effect than each modality used alone. Combined SU6668 and B7.2-IgG therapy resulted in complete eradication of pulmonary metastases in 50% of mice. Thus, antiangiogenic and immune therapies using SU6668 and B7.2-IgG fusion protein are compatible and manifest complementary antitumor and antimetastatic effects. Our results suggest that the combined attack against tumor cells and the tumor vascular system using antitumor immune mechanisms and antiangiogenic drugs can be a promising strategy for breast cancer therapy.

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THE N-TERMINAL FLANKING REGION OF THE MHC CLASS II INVARIANT CHAIN PEPTIDE AUGMENTS THE IMMUNOGENICITY OF A CRYPTIC "SELF" EPITOPE FROM THE HER-2/NEU TUMOR-ASSOCIATED ANTIGEN

Allan D. Hess, Christopher J. Thoburn, Weiran Chen, and Emilie C. Bright

Oncology Center, The Johns Hopkins University

adhess@jhmi.edu

The N-terminal flanking region of the MHC class II invariant chain peptide termed CLIP interacts with the T cell receptor at or near the binding site for bacterial superantigens, and therefore has superagonistic properties when presented by MHC class II. The present studies examine the hypothesis that the N-terminal segment of CLIP can augment the immunogenicity of cryptic "self" epitopes from tumor-associated antigens. Intradermal immunization with a chimeric construct of an MHC class II binding peptide (pp1171-1185) from the c-erb oncogene (Her-2/neu) containing the N-terminal flanking region of CLIP(-KPVSP(M-) elicits the induction of protective in vivo anti-tumor immunity against a Her-2/neu+ rat mammary tumor. Comparatively, the unmodified parent peptide was ineffective. The induction of effective anti-tumor immunity, however, requires either the presentation of the chimeric peptide on irradiated tumor cells or the peptide construct in concert with a MHC class I binding peptide (pp554-562) from Her-2/neu presented on dendritic cells. Interestingly, both immunization approaches result in the induction of potent CD8+ cytolytic T cell activity against unmodified tumor cells. As revealed by adoptive transfer studies, however, robust anti-tumor immunity in this setting requires both CD4+ and CD8+ T cells from animals immunized with the chimeric construct, findings that clearly implicate the importance of the CD4 T helper cell subset. In accord are the results from the in vitro analysis of CD4 T cell function post immunization. Immunization of animals with the parent Her-2/neu peptide results in a weak immune response to the unmodified peptide consisting of both type 1 (IL-2, IFN-gamma) and type 2 (IL-4, IL-10) cytokine producing cells as assessed by RT-PCR and by limiting dilution analysis. On the other hand, immunization with the chimeric Her-2/neu construct elicits a potent immune response to the parent, unmodified peptide with predominantly type 1 cytokine producing cells. Skewing of the responding T cell repertoire to type 1 cytokine producing cells appears to underlie, in part, the induction of protective anti-tumor immunity by immunization with the chimeric Her-2/neu peptide modified with the N-terminal sequence from CLIP.

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VACCINATION WITH A CARBOHYDRATE MIMOTOPE STIMULATES CELLULAR RESPONSES TO ESTABLISHED TUMOR

Behjatolah Monzavi-Karbassi, Ping Luo, Gina Cunto-Amesty, Marta Torres-Quinones, Fariba Jousheghany, and Thomas Kieber-Emmons

University of Pennsylvania

tom@xray.med.upenn.edu

Carbohydrate antigens are over-expressed on many tumor cells and efficient targeting of these antigens may broaden the tumor immunotherapies, leading to development of a broader category in malignant vaccines. However, carbohydrate antigens are typically poorly immunogenic, very difficult to purify in large quantities, difficult to synthesize, and usually induce mostly short-lived IgM type antibodies in vaccinated host without long lasting immunity. Among tumor associated carbohydrate antigens, the Histo-blood groups antigens are considered important targets for active specific immunotherapy targeting Breast cancer cells because of their correlation with prognosis. Two of these antigens, Lewis Y (LeY) and sialyl-Lewis X (sLeX), are especially relevant for cancer immunotherapy because of their broad expression. LeY and sLeX can form extended structures that are highly restricted to tumor cells. These antigens are intimately involved in adhesion and metastasis. There is no adequate in vivo tumor model for LeY since this antigen is not expressed on mouse tumor cells. In contrast, extended sLeX is expressed on mice fibrosarcoma cells providing an excellent animal model to evaluate both anti-tumor humoral and cellular responses. Carbohydrate determinants have traditionally not been considered as CTL targets as they are T cell independent antigens. We have recently reported that immunization with a peptide mimetic of LeY and extended sLex can enhance in vitro anti-tumor cellular responses. Here, we studied the effect of peptide mimotope immunization on established an murine tumor that expresses extended sLex. Immunization of mice with the peptide had a moderate effect on tumor regression. Inclusion of IL-12 in the immunization regimen stimulated complete elimination of tumor cells in all mice tested. As assessed by flow cytometry, we observed an increase in the T-cell proportion of splenocytes from immunized/cured mice as compared with non-immunized/tumor-bearing animals. Purified T cells from immunized/cured mice respond to IL-12 stimulation in vitro, as they produced IFN-g in a dose dependent manner. Adoptive transfer of immune T cells to tumor-bearing nude mice led to tumor eradication, indicating a role for CD8+ T cells in tumor regression.

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DIRECTING ANTI-GAL ANTIBODIES TO TARGET CELL SURFACES WITH SYNTHETIC LIGANDS

Robert Owen, Patricia Mowery, Yi He, and Laura L. Kiessling[1]

Departments of Chemistry and Biochemistry, University of Wisconsin-Madison, Madison, WI

kiessling@chem.wisc.edu

We are investigating the feasibility of directing a natural immune response to target cells involved in breast cancer using small molecules. Our strategy relies on the high concentrations of endogeneous antibodies that are naturally raised to the galactosyl-alpha(1̃3)-galactose [Gal-alpha(1-3)Gal] epitope. Our objective is to use a bifunctional small molecule that can simultaneously bind to the target cell surfaces and to the anti-gal antibodies. We envision that such a compound can direct the immune response toward breast cancer tumors. Studies in xenotransplantation have demonstrated that pig organs, which possess the Gal-alpha(1-3)Gal groups that are not found on primate or human cell surfaces, are rejected rapidly when implanted in primates. Approximately 1-3% of circulating antibodies recognize Gal-alpha(1-3)Gal, and thus, the immune system is poised to eliminate cells displaying this moiety. We are synthesizing small bifunctional molecules to target this response to breast cancer cells and the tumor vasculature. We hypothesize that these bifunctional, low molecular weight compounds will bind with high affinity to the surface of breast cancer cells (or the rapidly dividing endothelial cells important for the tumor blood supply), and this binding event will result in a multivalent display of the highly immunogenic epitope Gal-alpha(1-3)Gal. We anticipate that cells displaying high concentrations of this epitope will be eradicated. Our preliminary studies describing the synthesis of the bifunctional compounds, our preliminary results in cell culture, and our plans for improving their activities will be discussed.

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APPLICATION OF IMAGING APPROACHES TO THE MONITORING OF TARGETED THERAPIES OF BREAST CANCER

Bond Almand, MD, R. Michael Ahlquist, BS, David A. Mankoff, MD, Mary L. Disis, MD, and Keith L. Knutson, PhD

Division of Oncology, University of Washington,
Seattle, WA 98195

balmand@u.washington.edu

The development of targeted therapies has placed more emphasis on the need for *in vivo* imaging. The application of *in vivo* imaging techniques such as positron emission tomography (PET) can be used to monitor tumor-response to therapy, T cell homing, and the development of antigen-loss variants which will provide a better understanding of the mechanisms of therapeutic action. Here we describe the application of *in vivo* PET imaging techniques and other imaging methods to monitor response to targeted immunologic therapies in the neu-transgenic murine model of breast cancer. We first established an imaging model using standard doxorubicin chemotherapy to validate our ability to monitor tumor response to cytotoxic therapy. We developed a treatment regimen of repeated dosing of doxorubicin that halted tumor growth in all mice. Tumor metabolism and proliferation were measured using 3-H deoxyglucose and 14-C thymidine, respectively. Using a tissue harvesting imaging (i.e., cut and count) method, we demonstrated decreased tumor proliferation and metabolism, both of which correlated with changes in tumor size. We next applied PET imaging to this model using doxorubicin and observed significant decreases in tumor metabolism using ^{18}F-fluordeoxyglucose (FDG) PET imaging *in vivo*. This response was time-dependent and correlated with a decrease in tumor size. To better understand the *in vivo* specificity and activity of neu-specific adoptive T cell therapy, we are applying imaging techniques to track the homing of radio-labeled neu-specific T cells in tumor-bearing neu-transgenic mice. In our preliminary work we have demonstrated that radiolabeled neu-specific T cells retain antigen-specific activity as measured by ELIspot. We have shown decreased neu expression during the course of adoptive T cell therapy and stable neu expression with passive monoclonal antibody therapy. Using imaging to help understand the mechanisms of these targeted therapies and changes in antigen expression will be instructive for designing therapies capable of eradicating disease and developing combination therapies. The application of PET imaging techniques for biological-response monitoring is important for understanding many aspects of targeted therapies that can not be adequately addressed using *in vitro* correlates or size-based imaging techniques, such as CT. PET imaging is advantageous because it can be used to measure functional parameters of tumors that other methods of imaging can not. PET imaging can also provide better insight into the mechanisms of action of therapy.

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INHIBITION OF BREAST TUMOR GROWTH IN NEU-TRANSGENIC MICE WITH A MONOCLONAL ANTI-NEU ANTIBODY

Bond Almand, M.D., R Michael Ahlquist, B.S., Mary L Disis, M.D., and Keith L Knutson, Ph.D.

University of Washington Medical Center,
Seattle, WA 98195-6515

balmand@u.washington.edu

The use of monoclonal antibody therapy directed at tumor antigens for the treatment of solid tumors is well established. However, the mechanism of the tumor-antibody interaction is not well understood and has important implications for combinatory therapy with chemo- or other targeted therapies, such as neu-specific adoptive T cell therapy. Here we describe, in the neu-transgenic breast cancer model, the ability to inhibit and eradicate existing breast disease using monoclonal antibody therapy directed against the neu oncoprotein. We have developed a murine model of breast cancer using neu-transgenic mice implanted with neu-expressing mouse mammary carcinoma cells (MMC). Mice were treated with saline, anti-neu monoclonal antibody (7.16.4), or 7.16.4 plus doxorubicin at several timepoints after the subcutaneous injection of MMC. All saline-treated (control) mice developed palpable tumors within 8-10 days with 100% tumor take. However, when intravenous 7.16.4 treatment was started concurrently with tumor inoculation, all treated mice rejected the tumor challenge. Furthermore, the mice remained disease free after the cessation of 7.16.4 therapy, suggesting eradication. We have also demonstrated significant tumor inhibition when 7.16.4 therapy was started after palpable disease was present. The magnitude of the inhibition was dependent on the size of the tumors at the start of therapy, but growth of tumor still continued despite therapy. When doxorubicin chemotherapy was added in combination with 7.16.4, tumor growth was inhibited completely but regression was not observed. 7.16.4 therapy did not reduce neu antigen expression, as the tumors that were removed from treated mice from all groups demonstrated neu expression that was similar to that observed in control animals. The inhibition of growth without downregulation of neu expression was also seen in *in vitro* experiments which also demonstrated the retention of the 7.16.4 antibody on the cell surface. A possible explanation for the growth inhibition is that the 7.16.4 inhibits neu signaling, and ongoing studies will investigate this further. This data shows that neu-specific monoclonal antibody therapy results in tumor eradication or inhibition without the loss of cell surface antigen expression. The results also suggest that a major mechanism of action of neu antibody therapy is direct inhibition of neu-mediated growth. The maintenance of antigen expression and the diminution of the growth suggests that this therapy can be combined with other neu-targeted strategies, such as adoptive T cell therapy for improved treatment of neu-mediated malignancies.

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IDENTIFICATION OF HELPER PEPTIDES OF RAT NEU THAT CAN MEDIATE PARTIAL THERAPEUTIC ANTITUMOR RESPONSES IN BREAST TUMOR-BEARING NEU-TRANSGENIC MICE

Keith L. Knutson, Ph.D., R. Mike Ahlquist, B.S., and Mary L. Disis, M.D.

Division of Oncology, University of Washington,
Seattle, WA 98195

kknutson@u.washington.edu

T helper cells are pivotal to the development of both cell-mediated and humoral anti-tumor immunity. In this study we examined the phenotype and therapeutic efficacy of T cell lines specific for three putative MHC class II peptides derived from the intracellular domain of rat neu. The three sequences correspond to amino acids 781-795 (p781), 932-948 (p932), and 1166-1180 (p1166). Peptide-specific T cell lines could be generated from neu-tg mice that had been immunized with the peptides. The cell lines demonstrated peptide-specific proliferative activity. Lines against all 3 peptides also proliferated in response to neu+ tumor cells derived from neu-tg mice. The cell lines were predominantly $CD3^+/CD4^+/\alpha\beta$-TCR^+. The cell lines were of Th1 phenotype (i.e., IFN-g^+/IL-4^-). When T cell lines specific for p781 or p1166 were injected in tumor-bearing mice, there was a significant, albeit incomplete, inhibition of growth, suggesting therapeutic potential. The peptide-specific T cell lines derived from neu-tg mice are also being compared to peptide-specific T cell lines derived from the parental strain, FVB/N in order to identify how tolerance can interfere with therapeutic efficacy of the cells. Splenocytes derived from FVB/N parentals demonstrated significantly higher proliferation responses to p781 following immunization, suggesting that neu-tg mice have increased tolerance to this helper epitope. The observation that p781-specific T cell could be generated from neu-tg however indicates that p781-specific TCRs were retained in the neu-tg mouse TCR repertoire. Whereas T cells generated *ex vivo* from neu-tg mice predominantly CD4+/CD8-, the p781-specific T cells generated from FVB/N consisted of a large $CD8^+$ component. This suggested deletion of a $CD8^+$ responding T cell subset in the neu-tg mice. While the p781-specific T cell lines from both neu-tg and FVB/N were nearly equivalent in IFN-γ response as assessed by ELIspot analysis, the proliferative response of neu-tg T cells in response to tumor was greatly reduced compared to the response elicited by parental FVB/N. The identification of T cell helper epitopes of the neu oncoprotein may lead to improved strategies to modulate the immune response to prevent or treat neu-mediated tumors. Furthermore, understanding the tolerized phenotype of the tumor-responding T cells could potentially lead to the development of strategies aimed at correcting tumor-specific T cell non-responsiveness.

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LAYING THE FOUNDATION FOR T-CELL-MEDIATED IMMUNOTHERAPY IN A RAT MODEL OF BRAIN MICROMETASTASES

Lois A. Lampson, Ph.D., Rupa Kapoor, Yoichi Kondo, M.D., Ph.D., and Jennifer Durham

Department of Neurosurgery, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

lampson@rics.bwh.harvard.edu

INTRODUCTION. Metastasis to the brain is increasingly important as a cause of fatality in breast cancer patients. Yet few laboratories study brain metastases, and few models are available. It is especially important to be able to study the earliest micro-metastases that enter from the blood. Our previous work in other forms of brain micro-tumor provides a starting point. Our rationale is that migratory T cells are well-suited to reach and attack brain micro-tumor, including micro-metastases, whereas micro-tumor can't be easily reached or treated by other means. Our aims are to develop an appropriate small animal model, and to adapt T cell-mediated therapy to attack brain micro-metastases of mammary carcinoma.

METHODS, RESULTS, AND CONCLUSIONS.

1) First, we developed an appropriate, novel model. We adapted a commonly used rat mammary carinoma cell line, 13762 MAT BIII, by causing it to constitutively express the lacZ reporter gene product, b-galacotidase (b-gal). The b-gal marker aids detection of the smallest micro-metastases in tissue sections, and also serves as a well-defined tumor antigen. To obtain blood-borne metastases, we inject the tumor cells into the carotid artery of CDF rats, the same strain from which the cell line was derived. Thus, we have a syngeneic model for blood-borne brain metastases, appropriate for immunologic studies.

2) Our planned immunotherapy includes intracerebral injection of immune-enhancing cytokines. We showed that control injections of buffer do not change the pattern of tumor growth. This is an important control for our planned immunotherapy.

3) We compared TNF-a and IFN-g for their ability to enhance T cell surveillance. Graded doses of each cytokine were injected stereotactically into different brain regions. (Intracerebral injection avoids systemic toxicities.) Both cytokines enhance T cell entry, but IFN-g is more effective at activating phagocytes. Therefore, IFN-g was selected for use in further studies.

This work is important because brain metastases are increasingly important as a source of mortality for breast cancer patients, but few researchers study this topic, and a well-justified approach (T cell therapy) and an appropriate model were both needed.

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PROLIFERATION AND SURVIVAL AFTER SERIAL CYTOTOXICITY MEDIATED BY T CELLS ARMED WITH OKT3 X ANTI-HER2/NEU

Lawrence G. Lum, M.D., Ryan Grabert, Janelle Smith, and Nicola Kouttab, Ph.D.

Roger Williams Hospital, Providence, RI 02908

llum@rwmc.org

Nontoxic approaches are needed to improve cure rates in women with high risk breast cancer since chemotherapy is dose-limiting. Our strategy uses bispecific antibodies (BiAb) that target CD3 on activated T cells (ATC) and HER-2/neu (HER2) on breast cancer cells (SK-BR-3). Combining cellular and antibody-directed approaches may provide a greater anti-tumor effect than either alone. Armed ATC aggregate with SK-BR-3, mediate high levels of specific lysis, and produce IFNgamma, TNFalpha, and GM-CSF when exposed to HER2+ targets. ATC armed with anti-CD3 or anti-CD3 x anti-CD20 do not aggregate, enhance cytotoxicity, or synthesize cytokines when exposed to tumor. In this study, we asked whether armed ATC would survive and proliferate after cytotoxicity, and mediate cytotoxicity more than once. SK-BR-3 targets were plated overnight in a microcytotoxicity assay, labeled with 51-Cr and armed or unarmed ATC were added to microwells. Cytotoxicity assays wherein E:T ratios ranged from 2:1 to 10:1 were performed at 0, 45, 96, and 213 hours after arming of ATC with 50 ng of BiAb/million ATC. Lysis mediated by armed ATC was higher than unarmed ATC at all E:T ratios up to 96 hrs. Lyis at an E:T of 10:1 was 45, 25, 32, and 15% at 0, 45, 96, and 213 hrs, respectively. CSFE dye labeling prior to co-cultures of armed or unarmed ATC with SK-BR-3 tumor targets showed that the armed ATC divided several times during the repeated cycles of cytotoxicity. Armed ATC divided sooner than unarmed ATC exposed to SK-BR-3 and unarmed ATC alone. Immuno-fluoresence using goat anti-mouse antibody showed persistence of the BiAb on T cells 96 hrs after arming and lysis. Viability by trypan blue or propidium iodide showed mostly viable cells at 96 hrs. Phenotyping revealed both CD4 and CD8 cells. The armed T cells increased during culture. In summary, ATC kill multiple targets due to persistent BiAb on their surface, survive multiple lytic events without undergoing apoptosis after multiple exposures to tumor antigens, and proliferate after antigenic stimulation by tumor lysates. Infusions may induce clinical responses and immunize patients systemically.

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MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF)-RESPONSIVE T CELLS: POTENTIAL ROLE IN IMMUNOTHERAPY OF BREAST CARCINOMA

John Maher

Department of Immunology, King's College Hospital,
London SE5 9NU, UK

johnmaher_kcl@hotmail.com

Carcinoma of the breast is a highly lethal malignancy in which tumor cells produce M-CSF in the majority of cases. Aberrant M-CSF synthesis contributes to disease progression through enhancement of growth and invasiveness and also impaired differentiation of dendritic cells. The aim of this study is to genetically modify primary human T cells so that their growth, activation and effector function is enhanced by M-CSF. To achieve this, the c-fms-encoded human M-CSF receptor (M-CSFR) was first expressed in CD8+ (CTLL-2) and CD4+ (Jurkat) T cell lines. In CTLL-2 c-fms cells, M-CSF synergistically enhanced growth in response to limiting amounts of IL-2. Furthermore, M-CSF potently co-stimulated activation of these cells as measured by interferon gamma production. These actions of M-CSF are mediated by a pathway requiring Y809 of the M-CSFR and the Ras-MAP kinase cascade. Addition of M-CSF to Jurkat c-fms cells upregulates expression of CD69 but does not co-stimulate CD3-driven IL-2 production. However, when cultured on fibroblasts expressing the membrane-associated isoform of M-CSF, significant co-stimulation of IL-2 production was apparent. To investigate the functional activity of the M-CSFR in primary human T lymphocytes, cells were transduced with GALV-pseudotyped retroviral particles encoding c-fms. When activated using CD3, M-CSF synergistically enhanced proliferation, mimicking the co-stimulatory action of CD28 engagement. Upon sub-optimal stimulation with CD3 (1-100ng/ml) plus CD28 (1-100ng/ml), the further addition of M-CSF resulted in an up to 3 log increment in c-fms-expressing T cells over 9 days. Expansion of CD8+ T cells was 4-6 fold greater than that of CD4+ cells. By contrast, on-going experiments performed using purified T cell subsets have shown that the M-CSFR exerts a more potent co-stimulatory effect in the CD4+ subset. Taken together, these findings demonstrate the principle that T cells may be modified to receive potent co-stimulation of their growth and activation by a tumor-derived cytokine. In a clinical setting, this approach could be used to promote in vivo expansion of tumor specific T cells derived from the endogenous repertoire or whose antigenic specificity has been re-directed using an artificial T cell receptor.

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IDENTIFICATION OF HLA-A2-RESTRICTED T-CELL EPITOPES FROM MAMMAGLOBIN-A, A NEW BREAST CANCER-SPECIFIC ANTIGEN

Andrés Jaramillo, Kanchana Majunder, Timothy P. Fleming, Gerard Doherty, John F. Dipersio, and T. Mohanakumar

Departments of Surgery, Medicine, and Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

kumart@msnotes.wustl.edu

The mammaglobin-A gene encodes a glycoprotein that is highly over-expressed in human breast cancer (BC) cell lines and primary breast tumors. Since the pattern of expression of mammaglobin-A is restricted to mammary epithelium and metastatic breast tumors, the induction of mammaglobin-specific T cell immune responses may provide an important approach for designing specific tumor-targeted immunotherapy. Therefore, the purpose of this study was to identify HLA-A2-restricted mammaglobin-A-derived CD8+ T cell epitopes, and to determine whether CD8+ cytotoxic T lymphocytes (CTL) developed in vitro against mammaglobin-A-derived peptides have the capacity to kill BC cells naturally expressing mammaglobin-A.

A computer analysis of the mammaglobin-A amino acid sequence was performed to identify seven mammaglobin-A-derived peptides with the highest binding score to the HLA-A2 molecule (Mam-1-7). By means of ELISPOT analyses we determined that CD8+ T cells from BC patients (n=5) react to the Mam-1 (83-92), Mam-2 (2-10), Mam-4 (66-74), and Mam-7 (32-40) peptides and to a lesser extent to the Mam-3 (4-12) peptide. A CD8+ T cell line generated in vitro against the pool of mammaglobin-A-derived peptides showed significant HLA-A2-restrited cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative BC cells in vitro.

The results from this study indicate that mammaglobin is a breast cancer-associated antigenic protein. The use of HLA-A2-restricted mammaglobin-A epitopes could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

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IDENTIFICATION OF HLA-A3-RESTRICTED T-CELL EPITOPES FROM MAMMAGLOBIN-A, A NEW BREAST CANCER-SPECIFIC ANTIGEN

Andrés Jaramillo, Kanchana Majunder, Partha P. Manna, Timothy P. Fleming, Gerard Doherty, John F. Dipersio, and T. Mohanakumar

Departments of Surgery, Medicine, and Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

kumart@msnotes.wustl.edu

The mammaglobin-A gene encodes a glycoprotein that is highly over-expressed in human breast cancer (BC) cell lines and primary breast tumors. Since the pattern of expression of mammaglobin-A is restricted to mammary epithelium and metastatic breast tumors, the induction of mammaglobin-specific T cell immune responses may provide an important approach for designing specific tumor-targeted immunotherapy. Therefore, the purpose of this study was to determine whether mammaglobin-A-reactive CD4+ and CD8+ T cells are activated in vivo in BC patients, to identify HLA-A3-restricted mammaglobin-A-derived CD8+ T cell epitopes, and to determine whether CD8+ cytotoxic T lymphocytes (CTL) developed in vitro against mammaglobin-A-derived peptides have the capacity to kill BC cells naturally expressing mammaglobin-A.

By limiting dilution analysis, we determined that the frequency of mammaglobin-reactive CD4+ and CD8+ T cells in BC patients (n=7) is significantly higher than that observed in healthy female individuals (n=7) ($P = 0.025$ and $P = 0.029$, respectively). A computer analysis of the mammaglobin-A amino acid sequence was performed to identify eight mammaglobin-A-derived 9-mer peptides with the highest binding score to the HLA-A3 molecule (Mam-1-8). By means of ELISPOT analyses we determined that CD8+ T cells from BC patients (n=5) mainly react to the Mam-3 (2-10) and the Mam-4 (55-63) peptides and to a lesser extent to the Mam1 (23-31) and Mam-8 (58-66) peptides. A CD8+ T cell line generated in vitro against the pool of mammaglobin-A-derived peptides showed significant cytotoxic activity only to HLA-A3+ cells loaded with the Mam-1 peptide. This CD8+ T cell line displayed an HLA-A3-restrited cytotoxic activity against mammaglobin-A-positive but not mammaglobin-A-negative BC cells in vitro.

The results from this study indicate that mammaglobin is a breast cancer-associated antigenic protein. The use of HLA-A3-restricted mammaglobin-derived epitopes could provide a novel approach for designing new immunotherapy protocols for the treatment of breast cancer.

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MHC CLASS I MODULATION PROMOTES TUMOR ESCAPE FROM IMMUNOCYTOKINE-MEDIATED IMMUNE DESTRUCTION

Zane C. Neal and Paul M. Sondel

University of Wisconsin Comprehensive Cancer Center,
Madison, WI 53792

zcneal@facstaff.wisc.edu

Immunocytokines (IC) are monoclonal antibodies genetically linked to cytokines, such as IL2 or GM-CSF, which can target physiologically relevant concentrations of these biological response modifiers to the tumor microenvironment. Recently, it was demonstrated that huKS-IL2, an IL2-IC that binds to human epithelial cell adhesion molecule (EpCAM or KS), can induce an antitumor response against 4T1/KSA, a murine breast adnocarcinoma transfected to express huEpCAM. Although IC therapy has demonstrated potent responses against tumors like 4T1/KSA, CT26-EpCAM murine colon adenocarcinoma, and the NXS2 murine neuroblastoma, some tumors escape treatment and cause recurrent disease. To investigate tumor escape from IC therapy, we have chosen to pursue the NXS2 model, which circumvents the use of potentially strong immunogenic xenoantigens, like huEpCAM.

We have evaluated NXS2 tumors that recur following immunotherapy with hu14.18-IL2, a humanized IL2 IC targeted to the GD2 disialoganglioside that induces NK-dependent tumor resolution in mice. Hu14.18-IL2 therapy initially induced a clear antitumor response in all subcutaneous tumor-bearing mice, followed by delayed tumor recurrence in some mice. Importantly, we demonstrated that such recurrent NXS2 tumors exhibit enhanced (5-7 fold) MHC class I expression. This high H-2 phenotype appears dependent on selective pressures in vivo and was lost following brief culturing. Additionally, this enhanced H-2 expression on NXS2 cells was associated with reduced susceptibility to both NK cell-mediated tumor cell lysis and antibody-dependent cellular cytotoxicity in vitro. The combination of IL2-activated splenic NK cells, hu14.18-IL2, and NXS2 targets in vitro is sufficient to induce augmented MHC class I expression on NXS2 cells, which is abrogated in the presence of anti-mIFNg monoclonal antibody and implicates a role for IFNg in modulating tumor MHC class I expression.

Our demonstration that NXS2 tumors, in response to IC therapy, are capable of selectively increasing their MHC class I expression to escape NK-dependent tumor eradication, provides an investigational template to evaluate tumor escape in the 4T1/KSA model and other breast cancers, and illustrates the need for innovative approaches to circumvent tumor escape in vivo.

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COMPARISON OF THE IMMUNE RESPONSE IN TUMOR-FREE VERSUS TUMOR-CONTAINING SENTINEL LYMPH NODES OF BREAST CANCER PATIENTS

Nancy J. Poindexter, Ph.D., and Elizabeth A. Grimm, Ph.D.

The University of Texas M.D. Anderson Cancer Center, Houston, TX

npoindex@mdanderson.org

Sentinel lymph node (SLN) biopsy allows the identification of the major or first-draining lymph node from the site of a primary tumor. In breast cancer, the identification of tumor cells in these nodes is a predictor of the metastatic potential of the tumor. In this study we tested the hypothesis that the SLN is the primary site of antigen specific T cell activation. And, the activation state of the dendritic cell (DC), the major antigen presenting cell within the SLN, is predictive of the immune status as well as the nodal status of the tumor. Paraffin embedded SLN sections from breast cancer patients, including 25 tumor-free and 25 tumor-containing SLN, were analyzed by immunohistochemistry to determine the immune maturation state of the DC. Slides were stained with monoclonal antibodies against the following markers and cytokines: CD3, MHC class II, CD1a, CD83, IL-10, and IL-12. Mature DCs were defined by the expression of CD83 and high levels of MHC Class II; whereas immature DCs were defined as CD1a positive, CD83 negative and MHC Class II low. Our results demonstrated that tumor-free SLNs contained higher numbers of mature CD83+ DC compared to tumor-containing SLNs ($p=0.011$). These findings suggest that an active immune response was occurring in these nodes. In addition, tumor-free SLNs were more likely to contain cells expressing either IL-12 or IL-10 ($p=0.07$ and 0.009, respectively) compared to tumor-containing nodes.

The identification of immune activated, mature DCs in tumor-free nodes and the expression of both IL-10 and IL-12 suggests two possible immune mechanisms are occurring. The expression of IL-12 indicates T cell activation via a Th1 pathway while the presence of IL-10 is suggestive of immunosuppression via a Th2 response. Examination of the T cell responses in these patients will confirm whether T cell activation or suppression has occurred. This information will allow us to begin to dissect the functional components of a complete immune response that leads to tumor regression, thus allowing the development of successful immunotherapy.

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IMPROVEMENT OF MAMMARY TUMOR LOCALIZATION OF TRANSFERRED T CELLS THROUGH TRANSIENT INHIBITION OF T-CELL MYOSIN FUNCTION

Stuart Ratner, Jeffrey Oliver, and Jennifer Moreno

Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine, Detroit, MI

ratners@kci.wayne.edu

Introduction. Adoptive transfer of T cells is a potentially useful strategy for immunotherapy during immunosuppression, and for delivery of therapeutic vectors to tumor sites. Patient T cells are sensitized in vitro and infused back to the patient in an activated state. A serious limitation is the poor trafficking and tumor localization of these cells. The problem arises in part from the trapping and embolization of transferred T cells during their initial passages through pulmonary microvasculature. Damage done at this point causes the death of a large portion of the transferred cells, as evidenced by the accumulation of their debris in the liver and spleen. Pulmonary trapping is promoted by the active adhesion receptors and complex appendages induced in T cells by prolonged activation in vitro. We have found, however, that brief treatment with drugs which inhibit the function of the motor protein myosin can temporarily and without toxicity return activated T cells to the spherical, nonadhesive condition in which they normally circulate. We hypothesize the pulmonary trapping and destruction of activated T cells will be reduced, and their tumor homing increased, by treatment with a cocktail of myosin inhibitors (MI) prior to adoptive transfer.

Methods. The D2F2/E2 murine mammary tumor is a cell line engineered to express ErbB2, a common human breast cancer antigen. T cells from anti-ErbB2-vaccinated mice were specifically expanded and activated in vitro. They were labeled with the vital fluorescent dye CMFDA, treated with either saline or MI (125 uM ML-7 and 15mM butanedione monoxime), washed, and injected i.v. into syngeneic mice bearing s.c. D2F2E2 tumors, 2 X 10(7)/mouse. Mice were sacrificed at 0.5, 2, 24, and 48 hr and tumors, liver, spleen, and a set of peripheral lymph nodes were weighed, dispersed, and analyzed by flow cytometry for frequency of viable labeled cells. Known volumes of cell suspension were analyzed and results were normalized as number of labeled cells per gram of tissue.

Results. MI pretreatment caused an 8-fold reduction in short-term pulmonary localization ($p<0.05$). Mean localization at 0.5 hr for controls was 2.1 X 10(6) cells/g, whereas it was 0.25 X 10(6) cells/g for MI-pretreated cells. At 2 hr, there was still a significant (3-fold) difference. Localization of labeled cells in s.c tumors was significantly increased. At 24 hr it was 1.4 X 10(5)/g vs. 0.35 X 10(5)/g for controls. At 48 hr there the same 4-fold increase was seen in the MI condition. MI pretreatment also caused a small (20%) but significant increase in peripheral lymph nodes 48 hr.

Conclusions. The results support the hypothesis that transient myosin inhibition can reduce pulmonary trapping and improve tumor localization of infused activated T cells. If the increase in localization proves great enough to improve therapeutic effect, MI treatments could make adoptive immunotherapy a more useful weapon against breast cancer.

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FEASIBILITY OF LOW-DOSE CONTINUOUS INFUSION OF IL-2 AS A CONSOLIDATION TREATMENT FOLLOWING INTENSIVE BREAST CANCER CHEMOTHERAPY

W. E. Samlowski and F. B. Petersen

Huntsman Cancer Institute, University of Utah

wolfram.samlowski@hci.utah.edu

Patients with regionally advanced or metastatic breast cancer continue to have a high risk for death from metastatic disease despite the use of intensive chemotherapy. This may be due to persistence of chemotherapy resistant tumor cells. Interleukin-2 (IL-2) has been shown to induce cytolytic lymphocytes, termed lymphokine activated killer (LAK) cells that can kill multidrug resistant cancer cells in vitro. We therefore tested the feasibility of using a single course of low-dose continuous i.v. infusion of IL-2 as consolidation treatment after intensive cytoreductive chemotherapy. To test this concept, we performed a phase I/II trial in patients with limited metastatic breast cancer. Patients received the Stamp-V marrow ablative regimen, followed by stem cell rescue. In phase I, patients received continuous i.v. infusion of IL-2 (1.8 million IU/m2/d for18 days) starting either day 1 (5 patients) or day 14 (5 patients) following stem cell reinfusion. Endpoints evaluated included toxicity and LAK cell activation. We found that IL-2 infusions starting on day 1 resulted in a high frequency of febrile responses, along exacerbation of thrombocytopenia. A day 14 start was better tolerated with almost no detectable toxicity. Circulating lymphokine-activated killer cells could be detected in all patients, regardless of the day IL-2 treatment was initiated. To further assess clinical activity of this consolidation regimen, a total of 20 patients were treated with IL-2 consolidation beginning on day 14 post stem cell infusion (Phase II). Our data demonstrates that the majority of patients (85%) were able to complete the entire planned 18 day infusion of IL-2. Three patients ended the IL-2 infusion early due to severe malaise or rash. A total of 9/17 (45%) patients remained in clinical complete remission with a mean follow-up of >580 days (range 135-1175). Based on this data, we have initiated a clinical trial of IL-2 consolidation following doxorubicin/ cyclophosphamide plus paclitaxel adjuvant chemotherapy for high-risk regional breast cancer patients. Due to the high eventual risk of death due to breast cancer in this patient population, improved treatment strategies, such as IL-2 consolidation, are urgently needed.

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THE ARYL HYDROCARBON (DIOXIN) RECEPTOR AND CYTOCHROME P450 1B1 AS TARGETS FOR BREAST CANCER IMMUNOTHERAPY

Britta Maeker, Amanda Flies, Karen S. Anderson, Joachim L. Schultze, and David H. Sherr

Boston University School of Public Health

dsherr@bu.edu

Immunotherapy has long been viewed as an important approach to cancer treatment. Some recent clinical successes have fostered an air of renewed optimism among tumor immunologists. However, several obstacles to routine cancer immunotherapy, including the identification of appropriate tumor protein targets and the difficulty in breaking immune tolerance to "self" proteins expressed in tumors, remain. In the present studies we exploited recent advances in our ability to identify immunogenic peptides from "self" proteins and to present those peptides to the immune system of cancer patients to generate breast tumor-specific CD8+ killer T cell (CTL) responses in vitro. Two target proteins, the aryl hydrocarbon receptor/transcription factor (AhR) and the AhR-regulated cytochrome P450 CYP1B1 enzyme, were selected for these studies because of their high level expression in rodent and human breast tumors and their role in environmental chemical carcinogenesis. As a first step in generating killer T cell responses to tumors expressing these proteins, 3 computer-based algorithmes were used to identify AhR- and CYP1B1-derived peptides predicted to bind a relatively common human HLA class I allele, HLA-A*0201, with high affinity. HLA stabilization experiments with T2 cells confirmed high affinity binding of several AhR and CYP1B1 peptides to HLA-A*0201. Presentation of these peptides on dendritic cells and CD40 ligand-activated B cells to autologous human CD8+ T cells in vitro induced significant levels of cytotoxic CD8+ T cells (CTL) capable of lysing peptide-pulsed but not unpulsed HLA-A*0201 targets. Furthermore, these CTL killed tumors expressing AhR or CYP1B1 in an antigen- and HLA class I-specific manner. These CTL could be readily generated from cancer patients. CTL frequency analyses with fluorescent peptide/HLA*0201 tetramers indicated that between 1 and 4% of the CD8+ T cell population generated in vitro was responsible for the killing activity. Enrichment of the peptide/HLA*A0201-specific T cells by fluorescence activated cell sorting significantly enriched the killing activity. Collectively, the data demonstrate the feasibility of developing breast cancer vaccines with a platform that combines bioinformatics and advanced strategies for tumor antigen presentation.

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ORAL IMMUNIZATION: A NEW FRONTIER FOR BREAST CANCER PREVENTION

David A. Sirbasku

University of Texas Health Science Center - Houston

david.a.sirbasku@att.net

The goal of this laboratory and its affiliated Kaylone BioPharmaceuticals is to conquer breast cancer. One of the paramount steps in achieving this goal is to develop an oral immunization which can be administered to women of all ages to stimulate the natural secretory immune system to produce increased local tissue immunoglobulins such as IgA and IgM that negatively regulate breast epithelial cell growth and thereby prevent or substantially reduce the risk of breast cancer causing mutations. This study is the first step in establishing the feasibility of a new natural immune prevention. Our study is based on the well-established facts that in both experimental animals and human females there is a window during puberty/young adulthood in which breast tissue is most susceptible to genetic damage. Mutations during this period increase the incidence of breast cancer later in life. Furthermore, postmenopausal women may have another unrecognized high-risk window because the growth inhibitory potential of the secretory immune system diminishes as hormones decrease. We propose pre-clinical studies to establish that oral immunization of adolescent female rats can reduce breast tissue sensitivity to mutations. Second, we will alter the secretory immune system in adult and multiparous female rats to determine if there is another window later in life that also can be targeted by oral immunization. The most significant aspect of this study is that there is no previously known role for IgA and IgM as breast cancer cell growth regulators. Because IgA and IgM are negative regulators, and are increased in estrogen sensitive target tissues such as breast by oral antigen challenges, they are strong candidates to suppress the windows of rapid DNA synthesis in which mutations are more probable. This prevention proposal explores a highly adaptable means of stimulating a natural immune mechanism to prevent or reduce the risk of breast cancer worldwide. Today, no such oral immunization method exists for breast cancer.

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T-CELL RECOGNITION OF MONOCLONAL ANTIBODIES

Christopher M. Snyder, Xianghua Zhang, and Lawrence J. Wysocki

National Jewish Medical and Research Center

christopher.snyder@uchsc.edu

Monoclonal antibodies (mAbs) are attractive tools for the study, diagnosis and treatment of many types of cancer. A major hurdle to their use has been the immunogenicity of the antibodies themselves. Thus, much effort has been focused on "humanizing" the mAbs. However, many of these efforts have largely overlooked the immunogenicity of the antibodies from the perspective of the T cell repertoire. It is quite clear that T cell epitopes exist within monoclonal antibodies and that a few amino acid differences can often result in robust T cell reactivity. The result of this reactivity is not understood but recent work has suggested that anti-idiotypic antibodies may be ultimately be produced even in syngeneic mice.

We have devised an experimental system to study the T cell component of an immune response against a mAb. We developed a novel T cell receptor transgenic mouse in which greater than 98% of the T cells are specific for an epitope found in a particular monoclonal antibody. The epitope in question is located in the Vκ FR-1 region of a mAb (36-71) derived from a natural immune response. With these T cells we sought to develop a novel adoptive transfer model with which we could study the T cell response induced by passively administered antibody. In this model our goal was to create a scenario in which a mAb with a defined T cell epitope is administered to an adult patient. To this end, we adoptively transfer a small number of transgenic lymph node cells into recipient mice in order to follow the T cell responses in a physiological environment. Immunization of the recipient mice with the mAb or synthetic peptide induces a robust T cell response characterized by proliferation and cytokine production. Interestingly however, adoptive transfer of a small number of transgenic T cells followed by a tolerizing regiment of de-aggregated monoclonal antibody dampens the T cell response to subsequent immunizations with the antibody.

A T cell response to a foreign monoclonal antibody will likely provide help for an antibody response to the same mAb. In order to study T cells in this capacity we sought to extend our adoptive transfer model so that the kappa chain itself would be foreign. To this end, we have utilized kappa deficient mice as recipients for our adoptively transferred T cells. Thus, the B cells of the recipient mice can produce a robust antibody response to the kappa chain itself if they receive appropriate T cell help. With these tools we are currently studying the ability of the transgenic T cells to provide help for an anti-kappa antibody response in the kappa deficient mice. We can conclude from this and other work that T cells play an important role in the immune response induced by monoclonal antibodies.

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NOVEL METHOD TO STIMULATE TUMOR IMMUNITY

M. J. Leibowitz, J. Ma, and S. Stein

The University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School; Cancer Institute of New Jersey; and the Center for Advanced Biotechnology and Medicine

leibowit@umdnj.edu

Although the immune system plays a vital role in protection against cancer, as indicated by the increased incidence of cancer in various immunodeficiency states, attempts to develop a "cancer vaccine" have met with only limited success. Part of the limitation on this approach might be the variation in antigenic determinants on each patient's tumor, requiring a different vaccine for every patient. Furthermore, cancers appear to produce substances that inhibit immune system function, so that antigen presenting cells (APCs) fail to be activated when they come in contact with tumor cells. We are studying methods to inject tumors with depot formulations releasing immunostimulatory molecules, so that the patient can mount an immune response to the tumor. Using the MATIIIB tumor cell line in syngeneic Fischer 344 rats, we have tested the effect of injection of a polyethylene glycol-based hydrogel depot containing the immunostimulatory cytokine interleukin-12 (IL-12) and the APC chemoattractant peptide formyl-methionyl-leucyl-phenylalanine (fMLF) on tumor progression. In initial experiments, rats injected with one million tumor cells developed palpable tumors in about a week; the hydrogel was injected into the tumor on the first day it was palpable. Although this work is still in progress, preliminary data indicate that a single injection of both molecules causes a temporary suppression of tumor cell growth without significant toxicity. Work is continuing to determine the optimal formulation and injection schedule to inhibit tumor growth, and to determine whether the inhibition is due to enhanced recognition of the tumor cells by the immune system, as predicted.

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PROCATHEPSIN D STIMULATION OF HUMAN BREAST CANCER CELL GROWTH

V. Vetvicka

University of Louisville, Department of Pathology,
Louisville, KY 40202

vasek@louisville.edu

The long-term goal is to develop a new breast cancer treatment based on inhibition of the growth factor activity of procathepsin D. Based on our preliminary experiments showing that procathepsin D served as an autocrine mitogen, we prepared numerous clones of MDA-MD-231 and control cell line transfected with various human procathepsin D cDNA. First we demonstrated changes in production of procathepsin D by the transfected clones. After experiments establishing the basic values in invasiveness of parental cell line and the effect of procathepsin D, we showed that the invasiveness in vitro and metastatic properties in vivo is dependent on level of procathepsin D secretion. Further experiments showed that these effects are mediated by a structure within the activation peptide of procathepsin D. Detailed analysis using a library of synthetic peptides located the active moiety as position 36-44 of the activation peptide and showed that the activation of cancer cells involves interaction with a new cell surface receptor. Using flow cytometry analysis we found that no previously known surface antigens, soluble M6P-R or anti-M6P-R antibodies were able to inhibit the specific binding of pCD-FITC. Similarly, none of these substances inhibited growth factor activity of pCD. Using monoclonal antibodies raised against individual fragments of the activation peptide we demonstrated strong inhibition of both estradiol- and activation peptide-derived stimulation of in vitro proliferation of breast cancer cells. In addition, using an in vivo model of human breast cancer, we showed that injection of tumor-bearing mice with biodegradable microspheres containing anti-fragment or anti-activation peptide antibodies blocked the growth of breast cancer. The antibody-mediated protection from an induced breast cancer was almost complete for the whole tested interval of 8 weeks. Subsequent experiments showed that similar results were achieved with prostate cancer. In addition, mice immunized with activation peptide became resistant to breast cancer or prostate cancer challenge. Based on our data we propose that inhibition of procathepsin D synthesis and secretion represents a significant step towards inhibition of breast cancer.

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BOTH TYPES OF HERV-K HUMAN ENDOGENOUS RETROVIRUS ENV TRANSCRIPTS ARE SPLICED INTO SUBGENOMIC TRANSCRIPTS IN HUMAN BREAST CANCER

Feng Wang-Johanning,[1] Andra R. Frost,[2] Bixi Jian,[1] and Gary L. Johanning[3]

[1]Department of Medicine, [2]Department of Pathology, [3]Department of Nutrition Sciences

fengj@uab.edu

Human endogenous retroviruses (HERVs) are descendants of exogenous retroviruses that became cellular genes by integration into host germ line cells. Of the many HERV families, only HERV-K appears to have the full complement of open reading frames typical of replication competent mammalian retroviruses and comes closest of all HERVs to containing infectious virus. HERV-K has two types based on the presence or absence of an additional 292 bp sequence between the pol and env genes. In preliminary studies, we demonstrated that type 1 HERV-K env transcripts are expressed in most human breast cancer cells and tissues, but not in normal tissues. In the current study, we report that type 2 HERV-K env transcripts are also present in human breast cancer tissues and cell lines. Using RT-PCR, we found that both types of HERV-K env transcripts could be detected and were capable of being spliced into subgenomic env transcripts in the same breast cancer patients, with a great degree of complexity in the env transcripts present. Using real-time quantitative RT-PCR, we found that env transcriptional activity of HERV-K was 5 to 10 fold higher in HERV-K positive breast cancer cell lines treated with female hormone than in cells without treatment. Furthermore, the transcriptional activity of HERV-K env gene was higher in laser capture microdissected breast cancer cells than in uninvolved normal breast cells. The enhanced expression of HERV-K env gene in breast cancer cells may be specifically associated with female hormone activation. The selective expression and distribution of multiple HERV-K endogenous retroviral elements in human breast cancer, but not in normal breast tissues, suggests that they may perform significant biological role(s) in the host, and thus have been evolutionarily conserved.

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NOVEL PEPTIDE PRODRUG (PROPEPTIDE) DERIVED FROM IMMUNOGENIC HLA-A2.1-RESTRICTED FLU PEPTIDE MP58 GILGFVFTL IS ACTIVATED BY BETA-GLUCURONIDASE

Wei-Zen Wei, Sharad Rawale, Diego Laderach, Lew M. Hrihorczuk, and Jiri Zemlicka

Cancer Institute and [1]Department of Chemistry,
Wayne State University, Detroit, MI 48201

weiw@karmanos.org

The long term goal is to test the hypothesis that cancer can be eliminated by immunizing the host with foreign peptides followed by systemic delivery of pro-peptides which are activated at the tumor site by tumor associated enzymes. To test this hypothesis, HLA-A2.1 restricted flu peptide MP58 is linked at the N-terminus to beta-glucuronic acid which can be removed by beta-glucuronidase. Beta-glucuronidase is a 310-380 Kd lysosomal enzyme with exoglycosidase activity and is liberated from necrotic tumor cells and tumor infiltrating monocytes to create a high concentration of extracellular beta-glucuronidase in the tumor microenvironment. Beta-glucuronate was attached to the glycine moiety through a benzyloxycarbonyl linker. The product was reacted with octapeptide ILGFVFTL and the target prodrug beta-Glu-MP58 was generated following ester hydrolysis. Incubation of the prodrug with beta-glucuronidase triggers a self-destructive reaction to liberate free MP58 which was verified by HPLC. MP58 binds to HLA-A2.1 on human T2 cells in a dose dependent manner and A2.1 level increased by 230% in the presence of 60 mM MP58. When T2 cells were incubated with pro-peptide beta-Glu-MP58 up to 120 mM, their A2.1 level did not change. In the presence of 300 units/ml of beta-glucuronidase, A2.1 level increased by 210%, supporting the enzymatic liberation of active MP58 from the prodrug. An MP58 specific CTL line was established from peripheral blood monocytes of an A2.1 positive adult man by repeated stimulation with MP58 loaded antigen presenting cells. Over 80% of MP58 loaded T2 cells were lysed by CTL at E:T ratio of 10:1 – 2:1. The same CTL did not lyse T2 cells loaded with pro-peptide beta-Glu-MP58. In the presence of beta-glucuronidase, 80% of T2 cells were lysed, further demonstrating liberation of active MP58 from the prodrug to mark tumor cells for CTL lysis. These results demonstrate that immunogenic peptide prodrugs are novel agents with therapeutic potential for solid tumors.

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GENETIC IMMUNIZATION FOR BREAST CANCER

Max W. Sung, Shu-Hsia Chen, and Savio L. C. Woo

Mount Sinai School of Medicine, New York, NY 10029

max.sung@mssm.edu

Active specific immunotherapy with in vivo immunization using cytokine-expressing adenovirus vectors delivered by intratumoral injection in metastatic tumors may be effective in inducing antitumor immune responses and tumor regression in patients with metastatic breast cancer. Using an orthotopic model for hepatic metastases from breast cancer in syngeneic immunocompetent mice, we have shown that intratumora injection of a replication-defective adenovirus vector expressing murine interleukin-12 (ADV-mIL12) can deliver high concentrations of IL12 in the tumor microenvironment and induce effective local and systemic antitumor immune responses. Up to 40% of treated animals survived long term without disease relapse. No organ toxicities, systemic vector dissemination by PCR or elevation in serum proinflammatory cytokines (interleukin-6 and tumor necrosis factor alpha) were detected at therapeutically effective vector doses. To further enhance efficacy, we have combined intratumoral delivery of ADV-mIL12 with systemic delivery of an agonistic monoclonal antibody against 4-1BB, a T-cell costimulatory molecule, and found that up to 100% of treated animals in a hepatic metastasis model of colon cancer survived long term without evidence for residual disease, and developed long term innunity mediated by natural killer and T-cells.

We have translated these laboratory findings to a Phase I clinical trial with dose escalation of a replication-defecive adenovirus vector expressing human interleukin-12 (ADV-hIL12) in patients with hepatic metastases from breast cancer. To this end, we have constructed and produced ADV-hIL12, which after modification has passed lot release testing. The intratumoral vector injection is performed by percutaneous needle placement in a hepatic metastasis under concurrent ultrasonographic monitoring, a procedure we have tested in a previous clinical trial and found to be feasible and well tolerated. The ADV-hIL12 trial will be followed by a clinical trial of the agonistic monoclonal antiboody against 4-1BB delivered by intravenous infusion with dose escalation, combined with intratumoral ADV-hIL12 injection. Clinical translation of these immunotherapeutic approaches may lead to a new effective and safe treatment modality for breast cancer.

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A NOVEL BREAST CANCER ONCOGENE IS A VALIDATED TARGET FOR IMMUNOTHERAPY

Maurice Zauderer, Ph.D., Elizabeth E. Evans, Ph.D., Melinda A. Borrello, Ph.D., Alicia Henn, Ph.D., Ernest S. Smith, Ph.D., and Deepak M. Sahasrabudhe, M.D.

Vaccinex, Inc.

mzauderer@vaccinex.com

We have identified a novel gene/antigen, termed C35, that is highly overexpressed at the protein level in 70% of human breast carcinoma. C35 protein is undetectable in 29 of 31 normal tissues examined. Expression of C35 is detected in Leydig cells of testes, and, at relatively low levels, in some sections of normal breast epithelium. The pattern of C35 expression is compatible with use of C35 as a target for immunotherapy of breast cancer. In support of the immunogenicity of C35 for human T cells, we have successfully induced C35-specific primary human T cell responses in vitro by alternate stimulation with autologous dendritic cells infected with pox virus or retroviral recombinants of the C35 gene. To date, eight C35 peptides have been identified that are processed in tumors and presented to T cells in association with MHC class I molecules.

The C35 gene sequence has no homology to any known gene and its function is unknown. We have, however, determined that transfection of C35 into a normal breast epithelial cell line increases by a factor of 10 the number of colonies that form in soft agar. This is similar to results obtained for increased colony formation following transfection of the same cell line with the previously characterized ras oncogene and suggests that C35 has oncogenic activity.

An optimal target for immunotherapy of breast cancer should be differentially expressed in a large fraction of tumors relative to normal tissues. Further, in order to promote stable expression, the target protein should have a function associated with the tumor transformation phenotype. The properties of C35 suggest that it could serve as the basis for a broadly effective breast cancer vaccine.

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